(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

B3

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 May 2001 (25.05.2001)

PCT

(10) International Publication Number
WO 01/35979 A2

(51) International Patent Classification[7]: A61K 38/00

(21) International Application Number: PCT/US00/42137

(22) International Filing Date:
13 November 2000 (13.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/165,418 13 November 1999 (13.11.1999) US

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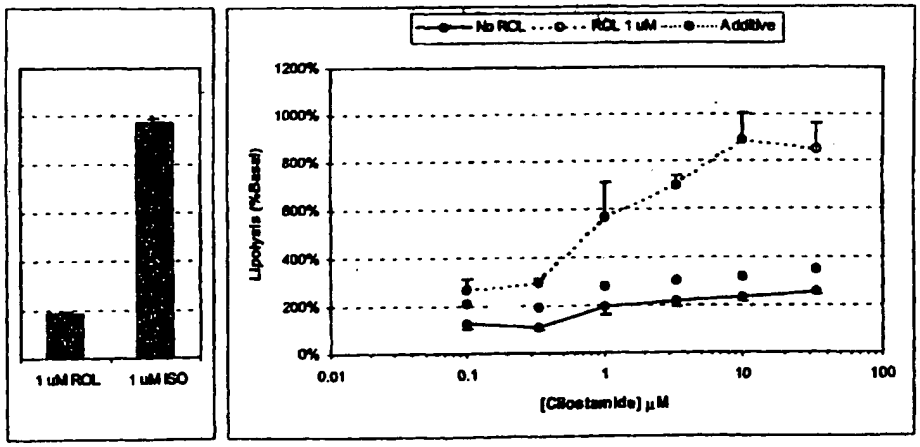
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMBINED PDE3 AND PDE4 INHIBITOR THERAPY FOR THE TREATMENT OF OBESITY



(57) Abstract: The present invention provides materials and methods for the treatment of obesity that involve a combination of a PDE3 and PDE4 inhibitor in synergistically effective amounts.

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Combined PDE3 and PDE4 Inhibitor Therapy for the Treatment of Obesity

FIELD OF THE INVENTION

The present invention relates to treatments for obesity.

BACKGROUND OF THE INVENTION

Obesity is a major risk factor for diabetes, hyperlipidemia, hypertension and coronary artery disease. In developed nations, the proportion of the population that is obese has been increasing in recent decades (reaching one third of the adult population of the United States in 1991) [Kuczmarski *et al., J. Amer. Med. Assoc., 272*: 205-211 (1995); Seidell *et al.* In: *Handbook of Obesity:* 79-91 (1998)]. In the U.S. alone, the cost of treating conditions linked to obesity has been estimated at more than $20 billion annually. The effectiveness of behavioral strategies (i.e. diet and exercise) in reducing weight is limited due to poor patient compliance. As a result, there is a need for pharmacotherapy as an adjunct for treatment of obese individuals with associated pathologies.

Current anti-obesity drugs are primarily appetite suppressants that work by potentiating the effect of satiety-inducing neurotransmitters in the central nervous system. They produce modest weight reduction in obese patients but can produce both cardiovascular and CNS side effects [Bray (1998) In: *Handbook of Obesity*: 953-975]. In addition, weight loss usually occurs only during the first three to six months of treatment, with no further loss observed even when drug treatment is continued for one year or more. This phenomenon is thought to result from an increase in energy efficiency following weight loss. According to this model, as body mass decreases, fewer calories need be consumed to sustain resting metabolic activity. As a result, energy expenditure eventually drops to match the reduced level of energy intake, and subsequently no further change in body weight occurs.

Thus, the development of pharmacological agents that increase resting metabolic rate is an important, but elusive, goal in anti-obesity research.

SUMMARY OF THE INVENTION

The present invention provides novel therapeutic materials and methods for the treatment of obesity.

For example, in one aspect, the invention provides a method of inducing or promoting or causing lipolyis in mammalian cells, comprising the steps of: contacting mammalian cells with a first compound that inhibits a phosphodiesterase 3 (PDE3) enzyme, and contacting the mammalian cells with a second compound that inhibits a phosphodiesterase 4 (PDE4) enzyme; wherein the first and second compounds are administered at concentrations that are effective to stimulate lipolysis in the cells. In a preferred embodiment, it is mammalian adipocytes that are contacted, preferably human adipocytes. For example, brown adipose cells or white adipose cells are contacted.

A compound is considered to be a PDE inhibitor compound if it effectively inhibits the phosphodiesterase activity of a PDE at a physiologically compatible concentration, or that is not overtly toxic to a cell at such concentration. Effective inhibition means that the inhibitor compound inhibits PDE activity by at least 30%, preferably at least 50%, more preferably at least 80%, and still more preferably at least 90%, at a physiologically compatible concentration.

Inhibition is typically measured using a dose-response assay in which a sensitive assay system is contacted with a compound of interest at a range of concentrations, spanning concentrations at which no or minimal effect is seen, through higher concentrations at which partial effect is seen, to saturating concentrations at which maximal effect is seen. Theoretically, such assays of the dose-response effect of inhibitor compounds can be described as a sigmoidal curve, expressing the degree of inhibition as a function of concentration. Such a curve will also theoretically pass through a point at which the concentration is sufficient to reduce activity of the enzyme to a level that is 50% that of the difference between

minimal and maximal enzyme activity in the assay, which is defined as the Inhibitory Concentration (50%) or IC_{50}. Comparisons of efficacy of inhibitors are often given with reference to comparative IC_{50} concentrations, in which a higher IC_{50} indicates that the test compound is less potent, and a lower IC_{50} indicates that the compound is more potent, than a reference compound.

Similarly, potency of compounds may be given in terms of the Effective Concentration (50%) or EC_{50}, which is a measure of dose-response activity in a cell-based or animal-based model. EC_{50} measurements are useful to indicate other properties of the compound that may influence its clinical utility such as the compound's solubility, its ability to penetrate cell membranes, its partition coefficient, its bioavailability, and the like. Note that two compounds may exhibit divergence in comparative IC_{50} and EC_{50} values, as one compound may be more potent in a biochemical assay and the other more potent in a cell-based assay simply due to different properties of the compounds.

Accordingly, a measure of comparative potency or selectivity is a ratio of IC_{50} (or EC_{50}) values for a compound with respect to two different enzymes. To illustrate, if the compound has an IC_{50} for enzyme A of 1 μM, and an IC_{50} for enzyme B of 10 μM, then the compound is said to have a 10-fold selectivity for enzyme A: IC_{50} B/ IC_{50} A = 10 μM / 1 μM = 10. In most cases, it is desirable that the selectivity of a compound be high such that, at an effective concentration for the target enzyme, the compound has minimal effect on other enzymes.

In one variation, the first compound selectively inhibits PDE3, and the second compound selectively inhibits PDE4; in a preferred variation, the first compound specifically inhibits PDE3 and the second compound specifically inhibits PDE4. For these purposes, a "selective" inhibitor compound is a compound that inhibits the indicated PDE activity (e.g., PDE3 or PDE4 activity) at least about 5-fold more effectively than the compound inhibits other members of the PDE family. Preferably, a selective inhibitor compound inhibits PDE3 and/or PDE4 at least about 10-fold, more preferably at least about 30-fold, more effectively than it inhibits other members of the PDE family. A "specific" inhibitor compound for these purposes is

defined as a compound that inhibits the indicated PDE activity (PDE3 or PDE4 activity) at least about 50-fold more effectively than it inhibits other members of the PDE family. Preferably, a specific inhibitor compound inhibits PDE3 and/or PDE4 at least about 100-fold, more preferably at least about 300-fold more effectively than it does other members of the PDE family. Selective and specific inhibitors are expected to promote lipolysis in adipocytes with fewer side-effects than non-selective inhibitors.

In a preferred embodiment, the first and second compounds are administered at synergistically effective concentrations. The term "synergistically effective concentrations" of two or more agents means concentrations wherein the desired effect (e.g., lipolyis) when administered together exceeds the additive effect observed when the same concentrations of the agents are used separately. Thus, for example, if 1 μM of a PDE3-inhibitor causes a 150% increase in lipolysis in adipocytes (when administered alone) and 1 μM of a PDE4 inhibitor causes a 125% increase in lipolyis, then a 275% increase when administered together would merely be considered additive, but a 500% increase when administered together would be considered synergistic. Likewise, if the PDE3 inhibitor caused a 50% increase in lipolytic activity and a PDE4 inhibitor alone caused no increase, but together the two inhibitors caused a 100% increase, then a synergistic effect is being observed. Preferred synergistic effects are 50% greater than additive effects. Highly preferred synergistic effects are 100%, 200%, 300%, 400% or 500% in excess of mere additive effects.

The PDE3 and PDE4 inhibitors can be administered either simultaneously, or sequentially. If administered sequentially, either the PDE3 inhibitor or the PDE4 inhibitor can be administered first.

Use of any PDE3 and PDE4 inhibitors are contemplated in the present invention. Known PDE3 inhibitors include amrinone, milrinone, cilostamide, anegralide, cilostazol, enoximone, piroximone, trequensin. Known PDE4 inhibitors include rolipram and CDP840. Additional exemplary PDE3 and PDE4 inhibitors are specifically identified below, and still additional inhibitors are known in the art and

are contemplated for use in the present invention. The examples below also provide assays for identifying novel inhibitors useful in the present invention.

The PDE3 enzyme exists in at least two isoforms, PDE3A and PDE3B. Since adipocytes apparently only express PDE3B to any significant extent, a PDE3 inhibitor that is selective or specific for PDE3B is highly preferred. Such an inhibitor can be selected using inhibition assays known in the art, such as the assay provided in the Example below.

In vivo methods of the invention are specifically contemplated. Thus, for example, the invention includes a method for inducing weight loss in a mammalian subject, comprising the steps of administering to the mammalian subject first and second compounds, wherein the first compound inhibits phosphodiesterase 3 (PDE3) enzyme and the second compound inhibits phosphodiesterase 4 (PDE4) enzyme, and wherein the compounds are administered at concentrations effective to promote weight loss in the mammalian subject. Administration to human subjects is specifically contemplated. Administration to chickens, turkeys, bovines (cows), porcines (pigs), deer, rabbits, and other animals that are commonly consumed as food also is contemplated. Treatment of humans who are clinically diagnosed as obese is specifically contemplated. Administration of synergistically effective concentrations of the inhibitors is preferred.

For *in vivo* methods of the invention, the PDE inhibitor compounds are preferably administered as one or more compositions wherein the compounds have been formulated with one or more pharmaceutically acceptable (*i.e.*, sterile and non-toxic) diluents, adjuvants, excipients, or carriers (e.g., water, saline solutions, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, glycerol, calcium phosphate, mineral oil, and cocoa butter). All forms of administration are contemplated, including but not limited to oral administration (e.g., as tables, solutions, or suspensions), topical administration, injection (e.g., intravenous, intramuscular, intraperitoneal), and the like.

Dosages for *in vivo* administration are extrapolated from doses effective to promote lipolysis *in vitro* in cell based assays (e.g., from IC_{50} and EC_{50} measurements). Dosages are selected to achieve similar circulating or cellular concentrations *in vivo* to doses that are effective *in vitro*. Dosages for *in vivo* administration are further determined using routine dose-response studies, first in animal models, and then in humans (after safety and efficacy has been established).

In addition to methods, the invention provides a composition comprising a first compound that inhibits phosphodiesterase 3 (PDE3) enzyme, in admixture with a second compound that inhibits phosphodiesterase 4 (PDE4) enzyme. Compositions that further include a pharmaceutically acceptable diluent, adjuvant, excipient, or carrier are specifically contemplated.

Similarly, the invention provides a kit for treatment of obesity comprising a first compound that inhibits phosodiesterase 3 (PDE3) enzyme, and a second compound that inhibits phosphodiesterase 4 (PDE4) enzyme; wherein the compounds are packaged together with instructions for administration of the compounds to a human subject to promote weight loss. In one variation, the first and second compounds are in admixture in a container, and wherein the instructions are provided on a label attached to the container. In another variation, the compounds are packaged together, but in separate vials, or separate tablets, or the like.

In still another variation, the invention is practiced with one or more compounds that act as both a PDE3 and a PDE4 inhibitor (e.g., zardaverine), preferably an inhibitor that is selective of specific for these two PDE's. For these purposes, a "selective" inhibitor compound is a compound that inhibits the two indicated PDE activities (PDE3 and PDE4 activity) at least about 5-fold more effectively than the compound inhibits other members of the PDE family.

For all methods of the invention, it will be appreciated that repetitive administration for ongoing treatment is specifically contemplated.

Additional features and variations of the invention will be apparent to those skilled in the art from the entirety of this application, and all such features are intended as aspects of the invention.

Likewise, features of the invention described herein can be re-combined into additional embodiments that also are intended as aspects of the invention, irrespective of whether the combination of features is specifically mentioned above as an aspect or embodiment of the invention. Also, only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the invention.

In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations specifically mentioned above. Although the applicant(s) invented the full scope of the claims appended hereto, the claims appended hereto are not intended to encompass within their scope the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 depicts the effects of the PDE3 inhibitor cilostamide on the level of lipolysis observed in a cultured mouse adipocyte cell line (3T3-L1), alone and in combination with 1 μM of the PDE4 inhibitor rolipram (ROL). Also shown are the lipolytic effects of 1 μM of rolipram alone and of the 1 μM of the β-adrenoreceptor agonist isoproterenol (ISO).

Figure 2 depicts the effects of the PDE3 inhibitor cilostamide on the level of lipolysis observed in cultured human adipocytes, alone and in combination with 1 μM of the PDE4 inhibitor rolipram (ROL). Also shown are the lipolytic effects

of 1 μM of rolipram alone and of the 1 μM of the β-adrenoreceptor agonist isoproterenol (ISO).

DETAILED DESCRIPTION

Ideally, anti-obesity pharmacological agents will increase resting metabolic rate, e.g., by stimulation of two processes: 1) hydrolysis of triglyceride stored in adipose tissue to glycerol and free fatty acids (lipolysis); and 2) oxidation of excess free fatty acids by a pathway coupled to the production of heat (thermogenesis) rather than to generation of ATP. Such lipolytic/thermogenic agents reduce metabolic efficiency by causing a greater fraction of the total caloric intake to be dissipated as heat rather than harnessed for useful cellular work. They thereby increase metabolic rate since more calories must be utilized to sustain basic cellular processes.

In the adipocyte, elevation of intracellular cAMP leads to stimulation of lipolysis and fatty acid oxidation. Hence, agents that cause such an elevation are candidates for anti-obesity therapeutics. Elevation of cAMP can be achieved either through stimulating cAMP synthesis (catalyzed by adenylyl cyclases) or through inhibiting cAMP degradation (catalyzed by cyclic nucleotide phosphodiesterases [PDEs]) [See Beavo, *Physiol. Rev., 75*: 725-748 (1995); Soderling *et al., Proc. Natl. Acad. Sci. USA 95*: 8991-8996 (1998); Soderling *et al., J. Biol. Chem., 273*: 15553-15558 (1998); Fischer *et al., J. Biol. Chem., 273*:15559-15564 (1998); Loughney *et al., Gene 234*: 109-117 (1999); Soderling *et al., Proc. Natl. Acad. Sci. USA, 96*: 7071-7076 (1999); and Fujishige *et al., J. Biol. Chem., 274*: 18438-18445 (1999), all incorporated herein by reference]. There are two primary cAMP-hydrolyzing PDEs in adipocytes: PDE3, which is primarily associated with the particulate fraction, and PDE4, which is the principal cytosolic cAMP-PDE in this cell type.

Two isoforms of PDE3 have been described: PDE3A and PDE3B, of which PDE3B is present in adipose tissues as well as hepatocytes, kidney epithelium, T cells, spermatocytes and embryonic neuroepithelium. [Taira *et al., J. Biol. Chem., 268*: 18573-18579 (1993); Reinhardt *et al., J. Clin. Invest., 95*: 1528-1538 (1995)]). A number of potent inhibitors that are selective for PDE3 are available. None of these

have been shown to possess significant selectivity for PDE3B over PDE3A. However, since only PDE3B has been detected in adipocytes, the effects of these agents on adipocytes can reasonably be attributed to inhibition of the PDE3B isoform.

Numerous studies in both isolated adipocytes and human subjects have documented the capacity of these PDE3 inhibitors to stimulate lipolysis. Results from these studies have also revealed differences between species in their sensitivity to PDE3 inhibitors. For example, the PDE3 inhibitor, cilostamide, has been shown to induce lipolysis and to potentiate the lipolytic effect of the β-adrenoreceptor agonist isoproterenol in murine 3T3-L1 adipocytes [Elks *et al., Endocrinol., 115*: 1262-1268 (1984)]. In isolated rat epididymal adipocytes, cilostamide also potentiates lipolysis under conditions of β-adrenergic stimulation, however, it has no significant effect on basal lipolysis. In the same cells, another PDE3 inhibitor, amrinone (Inocor™), has similar effects with that of cilostamide [Dorigo *et al., Biochem. Pharmacol., 38*: 855-858 (1989); and Schmitz-Peiffer *et al., Cell. Signal. 4*: 37-49 (1992)]. In human subjects, amrinone, in addition to stimulating lipolysis, also increases resting metabolic rate, as measured by O_2 consumption [Ruttimann *et al., Crit. Care Med. 22*: 1235-1240 (1994)]. Intravenous administration of amrinone in humans increased blood levels of glycerol and FFA, the breakdown products of triglyceride [Wilmshurst *et al., Br. Heart J. 52*: 38-48 (1984); Ruttimann *et al., Crit. Care Med. 22*: 1235-1240 (1994)]. Introduction of amrinone directly into the interstitial fluid of subcutaneous adipose tissue in humans using microdialysis, showed a dose-dependent increase in lipolysis as measured by the release of glycerol into the dialysate [Arner *et al., J. Lipid Res. 34*: 1737-1743 (1993)]. Amrinone is also able to antagonize the effect of insulin to inhibit lipolysis in adipocytes, and indicates that PDE3 inhibitors can render adipocytes insensitive to the anti-lipolytic action of insulin and thus promote continued breakdown of triglyceride [Hagstöm-toft *et al., Diabetes, 44*: 1170-1175 (1995); Moberg *et al., Horm. Metab. Res., 30*: 684-688 (1998)].

There are several inhibitors selective for PDE4, which can be categorized into three groups: xanthine derivatives, rolipram analogs, and quinazolinediones, and include benafentrine, tolafentrine, zardaverine, Org 20241,

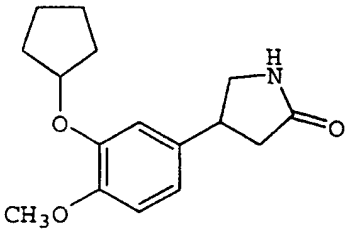
nitraquazone, RS 5344, BRL 1063, SB 207499, SDZ MKS 492, CDP 840, CP 80,633, RP 73401, WAY-PDA-641, LAS 31025, tibenelast, denbufylline, [Dent and Giembycz, in *Phosphodiesterase Inhibitors* (Schudt C, Dent G, Rabe KF, Eds.) Academic Press, San Diego. 1996, pp 111-126.], and substituted pyrrolidines [US Patent No. 5,665,754]. In contrast to PDE3 inhibitors, PDE4 inhibitors display comparatively little activity in lipolysis assays. Similar to results seen with inhibitors for PDE3, those specific for PDE4 also exhibit species-specific differences with respect to effects on lipolysis. For example, the PDE4-selective inhibitor, Ro-20-1724, stimulated lipolysis in murine 3T3-L1 cells to a lessor extent than the PDE3 inhibitor cilostamide and did not potentiate the effects of isoproterenol [Elks *et al.*, *Endocrinol., 115*: 1262-1268 (1984)]. In isolated rat epididymal adipocytes, Ro-20-1724 was capable of stimulating lipolysis, but only a concentration (300μM) at which it is no longer specific for PDE4 [Shechter, *Endocrinol., 115*: 1787-1791 (1984)]. Ro-20-1724 is also ineffective in stimulating lipolysis in primary human subcutaneous adipocytes [Kather and Scheurer, *Horm. Metabol. Res., 19*: 379-381 (1987)].

Several inhibitors of PDE3 and PDE4 have been described in the literature. See, e.g., Dent and Giembycz, in *Phosphodiesterase Inhibitors*, Schudt C, Dent G, Rabe KF, Eds., Academic Press, San Diego (1996), pp. 111-126; Crocker and Townley, *Drugs of Today, 35(7)*: 519-535 (1999), all incorporated herein by reference in their entirety.

Known PDE3 inhibitors include the following: Amrinone (5-amino-(3,4'-bipyridin)-6(1H)-one), commercially available from Sigma; Anegralide; Cilostamide; Cilostazol (6-[4-(1-cyclohexyl-1H-trazol-5-yl)-butoxy]-3,4-dihydro-2(1H) -quinolinone); Enoximone (1,3-dihydro-4-methyl-5-[4-(methylthio)benzoyl]-2H-imidazol-2-one); Milrinone (1,6-dihydro-2-methyl-6-oxo-(3,4'-bipyridine)-5-carbonitrile) (Sigma); Piroximone; Trequinsin (Sigma); and Siguazodan.

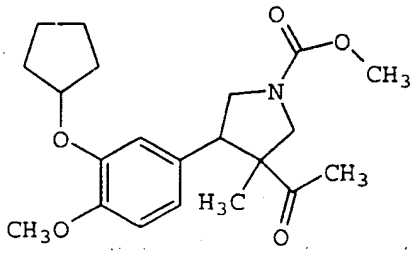
The drug rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone), an antidepressant agent commercially available from Sigma, was one of the first reported specific PDE4 inhibitors. Rolipram, having the following

structural formula, has a reported 50% Inhibitory Concentration (IC_{50}) of about 200 nM (nanomolar) with respect to inhibiting recombinant human PDE4.



Rolipram

Another class of PDE4 inhibitor compounds is disclosed in Feldman *et al.*, U.S. Patent No. 5,665,754, incorporated herein by reference. The compounds disclosed therein are substituted pyrrolidines having a structure similar to rolipram. One particular compound, having the following structural formula, has an IC_{50} with respect to human recombinant PDE4 of about 2 nM:



Among the compounds identified by Crocker and R.G. Townley (1999) as PDE4 inhibitirs in pre-clinical and clinical trials include RP-73401 (Rhone-Poulenc Rorer), D-22888 (Celtech); CP-80,633 (Pfizer), CDP-840 (Celtech), SB-207499 (SmithKline Beecham), AWD-12-281 (Celtech), and D-4418 (Chroscience/Schering Plough. These and other compounds have been described in the literature and are synthesized using conventional methods.

Compounds that inhibit both PDE3 and PDE4 also have been described in the literature, including zardaverine and isobutylmethylxanthine (IBMX).

In addition, the examples below provide assays to identify additional PDE3 and PDE4 inhibitors.

The invention will now further be desribed by the following examples, which are intended as exemplary and not limiting in their description of the invention.

Example 1

Synergistic Lipolytic effects of PDE3 and PDE4 inhibitors

The following experiments demonstrated that PDE3 and PDE4 inhibitors act synergistically to promote lipolysis in mammalian adipocytes, providing evidence of their utility (in combination) for treating weight problems, such as obesity.

In a first set of experiments, the lipolytic effects of PDE3 and PDE4 inhibitors were assayed in cultured murine adipocytes (3T3-L1 cells). As set forth below, the PDE4 inhibitor was found to dramatically potentiate the ability of the PDE3 inhibitor to induce lipolysis in these cells. The Experimental results were then extended to cultured human adipocytes.

Experimental Protocols

A. Culture of Murine 3T3-L1 cells

A murine fibroblastic cell line (3T3-L1 cells) that can be induced to differentiate into adipocytes *in vitro* was obtained from American Type Culture Collection. Cells are grown in 24-well tissue culture plates in a growth medium consisting of: Dulbeco's Minimal Essential Medium (Gibco) supplemented with 25 mM glucose, 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. Cells are maintained at 37°C in an atmosphere containing 5% CO_2. Differentiation was carried out according to the method of Vasta *et al.*, *Biochem. Biophys. Res. Commun. 183*: 1070-1075 (1992). Briefly, when the cells have reached confluence, they are incubated for three days in growth medium supplemented with 0.4 µM dexamethasone, 500 µM Isobutylmethylxanthine and 10 µg/ml bovine insulin (all from Sigma). They are then incubated for a further three days in growth medium

supplemented only with 10 μg/ml bovine insulin. Finally, they are shifted back to standard growth medium for three days. At the end of this procedure, > 90% of the cells have differentiated into adipocytes (as evidenced by the intracellular accumulation of lipid). Differentiated cells are maintained in growth medium for up to 10 days, with fresh medium supplied every two to three days.

B. Lipolysis Assay:

Lipolysis is measured by the accumulation of glycerol (a breakdown product of triglyceride) in the culture medium. The following protocol is employed.

Confluent monolayers of adipocytes are washed with Dulbeco's PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$) and then treated with inhibitors that have been diluted to the appropriate concentration in a diluent consisting of Dulbeco's Minimal Essential Medium + 25 mM glucose (w/o FBS). Inhibitors stocks are dissolved in 100% DMSO; therefore, a vehicle control containing the same concentration of DMSO as in the drug dilutions is also tested. Each test condition is done in triplicate. Cells are incubated with inhibitors or vehicle for 6 hours at 37°C in an atmosphere containing 5% CO_2. Supernatants from the treated cells are collected and stored at -20°C until ready for analysis.

Measurement of glycerol in the culture supernatant is carried out in a 96-well format as follows: 25 μl of each supernatant is combined with 75 μl of diluent and mixed with 100 μl of GPO-Trinder glycerol assay reagent (Sigma). The reaction is allowed to proceed for 5 minutes at room temperature and the absorbance of the sample is determined at 540 nm. The value of a blank well (containing only medium) is subtracted from each well. The mean and the standard error of the mean are determined for each set of three replicates and these values are expressed as a percentage of the value for the vehicle control.

C. Experiment with human subcutaneous adipocytes:

Human adipocyte experiments were done by Zen-Bio, Inc. (Research Triangle Park, NC) under blind contract (whereby the investigators were not informed of the identity of the compounds that were being tested). Human adipocyte precursor cells are derived from surgical specimens of subcutaneous adipose tissue by collagenase digestion. These cells are grown to confluence in 96-well tissue culture plates and induced to differentiate into adipocytes in medium containing adipogenic and lipogenic hormones. (See Hauner *et al.*, *J. Clin. Invest. 84*: 1663-1670 (1989) for a representative protocol.) Treatment of cells with inhibitors and determination of glycerol levels in the culture supernatant are performed essentially as described above for 3T3-L1 cells.

Results

The PDE3 inhibitor cilostamide was found to dose-dependently stimulate lipolysis in 3T3-L1 cells with a maximal effect of about 2.5-fold at the highest concentration tested (33 μM). The PDE4 inhibitor rolipram was found to stimulate lipolysis about 1.9-fold at 1 μM, and higher concentrations did not have a greater effect.

When various concentrations of cilostamide were tested in combination with 1 μM rolipram, the observed stimulation of lipolysis was far greater than expected from a simple additive effect of the two agents. The combination of 1 μM rolipram and a maximally effective concentration of cilostamide (10 μM) stimulated lipolysis by approximately nine-fold above basal levels. This response was similar to that observed when cAMP production by adenylyl cyclase is maximally stimulated with a β-adrenoreceptor agonist (1 μM isoproterenol). These results are summarized in Figure 1. Previous investigators failed to observe a synergistic effect between the PDE3 inhibitor cilostamide and a PDE4 inhibitor Ro-20-1724. See Elks and Manganiello, *Endocrinol. 115*: 1262-1268 (1984).

In a related set of experiments, the effect of PDE3 and PDE4 inhibitors on lipolysis in cultured human adipocytes was examined.

A bi-phasic response to cilostamide was observed in the human adipocytes, with a maximal stimulation (2.3-fold) at 1 μM and progressively lower stimulation at higher concentrations of cilostamide. (See Fig. 2.) This response pattern may reflect toxicity of cilostamide to these cells. Rolipram (1 μM) alone had no observable effect on lipolysis.

When cilostamide and rolipram were combined, a potentiation of the lipolytic response was observed, which was most noticeable at higher concentrations of cilostamide (Fig. 2.). In the presence of rolipram, there was a less pronounced drop-off of activity with increasing cilostamide concentration.

Unlike the murine 3T3-L1 cells, the human adipocytes used in this study were not a clonal population, and the human cells displayed a higher level of variability in their lipolytic response. It is also noteworthy that the human cells are less responsive to the β-adrenergic agonist isoproterenol (1.4-fold stimulation at 1 mM) than they are to cilostamide. It is therefore possible that the maximal stimulation of these cells is smaller than it is for 3T3-L1 cells (nearly 10-fold, see Fig. 1).

In addition to a synergistic effect in the treatment of obesity, the combination PDE3 inhibitor/PDE4 inhibitor therapy is expected to have at least two potential advantages over PDE3 inhibition alone. First, it is expected that combined therapy will produce fewer cardiac side effects than a PDE3 inhibitor alone, because the desired therapeutic effect will be achieved with lower doses of the PDE3 inhibitor. This follows from the observation that PDE3 is expressed in cardiovascular tissues. Commonly used PDE3 inhibitors have cardiotonic properties that include positive inotropy (increased force of cardiac contraction) and peripheral vasodilation. It is therefore possible that the potentiating effects of a co-administered PDE4 inhibitor will allow the use of a lower dose of PDE3 inhibitor, with fewer attendant cardiovascular side effects.

Second, combined therapy may have an anti-diabetic effect by virtue of the effect of PDE4 inhibitors on TNFα secretion. TNFα has been implicated in the development of the insulin-resistance that is seen in many obese,

non-insulin-dependent diabetics. In vitro, TNFα inhibits the insulin receptor tyrosine kinase. Furthermore, TNFα levels are elevated in adipose tissue of obese animals and neutralization of TNFα improves the insulin-responsiveness of obese animals. Therefore, PDE4 inhibitors, being effective antagonists of TNFα secretion, may improve insulin sensitivity in obese, insulin resistant subjects.

Example 2

Production of PDE Proteins

The following protocols are useful for producing phosphodiesterase proteins. The proteins are useful, for example, in assays for PDE activity and assays to identify PDE inhibitors.

A. Expression in Baculovirus-Infected *Spodoptera fugiperda* (Sf9) Cells

Baculovirus transfer plasmids were constructed using either pBlueBacIII (Invitrogen) or pFastBac (BRL-Gibco). The structure of all plasmids was verified by sequencing across the vector junctions and by fully sequencing all regions generated by PCR. Plasmid pBB-PDE3A contained the complete open reading frame of PDE3A (Meacci *et al., Proc. Natl. Acad. Sci. USA 89*: 3721-3725 (1992)) in pBlueBacIII. Plasmid pFB-PDE3B contained the complete open reading frame of PDE3B (Miki *et al., Genomics 36*: 476-485 (1996)) in pFastBac.

Recombinant virus stocks were produced using either the MaxBac® system (Invitrogen) or the FastBac™ system (Gibco-BRL) according to the manufacturer's protocols. In both cases, expression of recombinant human PDEs in the resultant viruses was driven by the viral polyhedron promoter. When using the MaxBac® system, virus was plaque purified twice in order to insure that no wild type (occ+) virus contaminated the preparation. Protein expression was carried out as follows. Sf9 cells were grown at 27°C in Grace's Insect culture medium (Gibco-BRL) supplemented with 10% fetal bovine serum, 0.33% TC yeastolate, 0.33% lactalbumin hydrolysate, 4.2 mM $NaHCO_3$, 10 μg/mL gentamycin, 100 units/mL penicillin, and 100 μg/mL streptomycin. Exponentially growing cells were infected at a multiplicity

of approximately 2 to 3 virus particles per cell and incubated for 48 hours. Cells were collected by centrifugation, washed with nonsupplemented Grace's medium, and quick-frozen for storage.

B. Expression in *Saccharomyces cerevisiae* (Yeast)

Recombinant production of human PDE4A, PDE4B and PDE4C, PDE4D was carried out similarly to that described in Example 7 of U.S. Patent No. 5,702,936, incorporated herein by reference, except that the yeast transformation vector employed, which is derived from the basic ADH2 plasmid described in Price *et al., Methods in Enzymology, 185*: 308-318 (1990), incorporated yeast ADH2 promoter and terminator sequences and the *Saccharomyces cerevisiae* host was the protease-deficient strain BJ2-54 deposited on August 31, 1998 with the American Type Culture Collection, Manassas, Virginia, under accession number ATCC 74465. Transformed host cells were grown in 2X SC-leu medium, pH 6.2, with trace metals, and vitamins. After 24 hours, YEP medium-containing glycerol was added to a final concentration of 2X YET/3% glycerol. Approximately 24 hours later, cells were harvested, washed, and stored at -70°C.

C. Preparation of PDE3A from Sf9 Cells

Cells (2 X 10^{10}) were suspended in Lysis Buffer containing 50 mM MOPS, pH 7.5, 2 mM DTT, 2 mM benzamidine HCl, 5 µM $ZnSO_4$, 0.1 mM $CaCl_2$, 20 µg/mL calpain inhibitors I and II, and 5 µg/mL each of leupeptin, pepstatin, and aprotinin. The mixture was sonicated twice for 30 seconds and the cells were lysed in a French® pressure cell (SLM-Aminco®, Spectronic Instruments) at 4°C. The lysate was centrifuged 100,000 X g for 45 minutes. The pellet was washed once in Lysis Buffer and suspended in 46 mL Lysis Buffer with a Dounce homogenizer. Aliquots were stored at -70°C. These preparations had specific activities of about 1 to 2 nmol cAMP hydrolyzed per minute per milligram protein.

D. Preparation of PDE4A from *S. cerevisiae*

Yeast cells (50 g of yeast strain Y126 harboring HDUN1.46) were thawed at room temperature by mixing with 50 mL of Lysis Buffer (50 mM MOPS pH 7.5, 10 μM $ZnSO_4$, 2 mM $MgCl_2$, 14.2 mM β-mercaptoethanol, 5 μg/mL each of pepstatin, leupeptin, aprotinin, 20 μg/mL each of calpain inhibitors I and II, and 2 mM benzamidine HCl). Cells were lysed in a French® pressure cell (SLM-Aminco®, Spectronic Instruments) at 10°C. The extract was centrifuged in a Beckman JA-10 rotor at 9,000 rpm for 22 minutes at 4°C. The supernatant was removed and centrifuged in a Beckman TI45 rotor at 36,000 rpm for 45 minutes at 4°C.

PDE4A was precipitated from the high-speed supernatant by the addition of solid ammonium sulfate (0.26 g/mL supernatant) while stirring in an ice bath and maintaining the pH between 7.0 and 7.5. The precipitated proteins containing PDE4A were collected via centrifugation in a Beckman JA-10 rotor at 9,000 rpm for 22 minutes. The precipitate was resuspended in 50 mL of Buffer G (50 mM MOPS pH 7.5, 10 μM $ZnSO_4$, 5 mM $MgCl_2$, 100 mM NaCl, 14.2 mM β-mercaptoethanol, 2 mM benzamidine HCl, 5 μg/mL each of leupeptin, pepstatin, and aprotinin, and 20 μg/mL each of calpain inhibitors I and II) and passed through a 0.45 μm filter.

The resuspended sample (50 to 100 mL) was loaded onto a 5 X 100 cm column of Pharmacia Sephacryl® S-300 equilibrated in Buffer G. Enzyme activity was eluted at a flow rate of 2 mL/min. and pooled for later fractionation.

The PDE4A isolated from gel filtration chromatography was applied to a 1.6 X 20 cm column of Sigma Cibacron Blue Agarose-type 300 (10 mL) equilibrated in Buffer A (50 mM MOPS pH 7.5, 10 μM $ZnSO_4$, 5 mM $MgCl_2$, 14.2 mM β-mercaptoethanol, and 100 mM benzamidine HCl). The column was washed in succession with 50 to 100 mL of Buffer A, 20 to 30 mL of Buffer A containing 20 mM 5'-AMP, 50 to 100 mL of Buffer A containing 1.5 M NaCl, and 10 to 20 mL of Buffer C (50 mM Tris HCl pH 8, 10 μM $ZnSO_4$, 14.2 mM β-mercaptoethanol, and 2 mM benzamidine HCl). The enzyme was eluted with 20 to 30 mL of Buffer C containing 20 mM cAMP.

The PDE activity peak was pooled and precipitated with ammonium sulfate (0.33 g/mL enzyme pool) to remove excess cyclic nucleotide. The precipitated proteins were resuspended in Buffer X (25 mM MOPS pH 7.5, 5 μM $ZnSO_4$, 50 mM NaCl, 1 mM DTT, and 1 mM benzamidine HCl), and desalted via gel filtration on a Pharmacia PD-10® column per manufacturer's instructions. The enzyme was quick-frozen in a dry ice/ethanol bath and stored at -70°C.

The resultant preparations were about >80% pure by SDS-PAGE. These preparations had specific activities of about 10 to 40 μmol cAMP hydrolyzed per minute per milligram protein.

E. Preparation of PDE4B from *S. cerevisiae*

Yeast cells (150 g of yeast strain YI23 harboring HDUN2.32) were thawed by mixing with 100 mL glass beads (0.5 mM, acid washed) and 150 mL Lysis Buffer (50 mM MOPS pH 7.2, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 2 mM benzamidine HCl, 5 μg/mL each of pepstatin, leupeptin, aprotinin, calpain inhibitors I and II) at room temperature. The mixture was cooled to 4°C, transferred to a Bead-Beater®, and the cells lysed by rapid mixing for 6 cycles of 30 seconds each. The homogenate was centrifuged for 22 minutes in a Beckman J2-21M centrifuge using a JA-10 rotor at 9,000 rpm and 4°C. The supernatant was recovered and centrifuged in a Beckman XL-80 ultracentrifuge using a TI45 rotor at 36,000 rpm for 45 minutes at 4°C. The supernatant was recovered and PDE4B was precipitated by the addition of solid ammonium sulfate (0.26 g/mL supernatant) while stirring in an ice bath and maintaining the pH between 7.0 and 7.5. This mixture was then centrifuged for 22 minutes in a Beckman J2 centrifuge using a JA-10 rotor at 9,000 rpm (12,000 X g). The supernatant was discarded and the pellet was dissolved in 200 mL of Buffer A (50 mM MOPS pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine HCl, and 5 μg/mL each of leupeptin, pepstatin, and aprotinin). The pH and conductivity were corrected to 7.5 and 15-20 mS, respectively.

The resuspended sample was loaded onto a 1.6 X 200 cm column (25 mL) of Sigma Cibacron Blue Agarose-type 300 equilibrated in Buffer A. The sample

was cycled through the column 4 to 6 times over the course of 12 hours. The column was washed in succession with 125 to 250 mL of Buffer A, 125 to 250 mL of Buffer A containing 1.5 M NaCl, and 25 to 50 mL of Buffer A. The enzyme was eluted with 50 to 75 mL of Buffer E (50 mM Tris HCl pH 8, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 2 mM benzamidine HCl, and 20 mM cAMP) and 50 to 75 mL of Buffer E containing 1 M NaCl. The PDE activity peak was pooled, and precipitated with ammonium sulfate (0.4 g/mL enzyme pool) to remove excess cyclic nucleotide. The precipitated proteins were resuspended in Buffer X (25 mM MOPS pH 7.5, 5 μM $ZnSO_4$, 50 mM NaCl, 1 mM DTT, and 1 mM benzamidine HCl) and desalted via gel filtration on a Pharmacia PD-10® column per manufacturer's instructions. The enzyme pool was dialyzed overnight against Buffer X containing 50% glycerol. This enzyme was quick-frozen in a dry ice/ethanol bath and stored at -70°C.

The resultant preparations were about >90% pure by SDS-PAGE. These preparations had specific activities of about 10 to 50 μmol cAMP hydrolyzed per minute per milligram protein.

F. Preparation of PDE4C from *S. cerevisiae*

Yeast cells (150 g of yeast strain YI30 harboring HDUN3.48) were thawed by mixing with 100 mL glass beads (0.5 mM, acid washed) and 150 mL Lysis Buffer (50 mM MOPS pH 7.2, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 2 mM benzamidine HCl, 5 μg/mL each of pepstatin, leupeptin, aprotinin, calpain inhibitors I and II) at room temperature. The mixture was cooled to 4°C, transferred to a Bead-Beater®, and the cells lysed by rapid mixing for 6 cycles of 30 seconds each. The homogenate was centrifuged for 22 minutes in a Beckman J2-21M centrifuge using a JA-10 rotor at 9,000 rpm and 4°C. The supernatant was recovered and centrifuged in a Beckman XL-80 ultracentrifuge using a TI45 rotor at 36,000 rpm for 45 minutes at 4°C.

The supernatant was recovered and PDE4C was precipitated by the addition of solid ammonium sulfate (0.26 g/mL supernatant) while stirring in an ice bath and maintaining the pH between 7.0 and 7.5. Thirty minutes later, this mixture

was centrifuged for 22 minutes in a Beckman J2 centrifuge using a JA-10 rotor at 9,000 rpm (12,000 X g). The supernatant was discarded and the pellet was dissolved in 200 mL of Buffer A (50 mM MOPS pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 2 mM benzamidine HCl, and 5 µg/mL each of leupeptin, pepstatin, and aprotinin). The pH and conductivity were corrected to 7.5 and 15-20 mS, respectively.

The resuspended sample was loaded onto a 1.6 X 20 cm column (25 mL) of Sigma Cibacron Blue Agarose-type 300 equilibrated in Buffer A. The sample was cycled through the column 4 to 6 times over the course of 12 hours. The column was washed in succession with 125 to 250 mL of Buffer A, 125 to 250 mL of Buffer A containing 1.5 M NaCl, and then 25 to 50 mL of Buffer A. The enzyme was eluted with 50 to 75 mL of Buffer E (50 mM Tris HCl pH 8, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 2 mM benzamidine HCl, and 20 mM cAMP) and 50 to 75 mL of Buffer E containing 1 M NaCl. The PDE4C activity peak was pooled, and precipitated with ammonium sulfate (0.4 g/mL enzyme pool) to remove excess cyclic nucleotide. The precipitated proteins were resuspended in Buffer X (25 mM MOPS pH 7.2, 5 µM $ZnSO_4$, 50 mM NaCl, 1 mM DTT, and 1 mM benzamidine HCl) and desalted via gel filtration on a Pharmacia PD-10® column per manufacturer's instructions. The enzyme pool was dialyzed overnight against Buffer X containing 50% glycerol. This enzyme was quick-frozen in a dry ice/ethanol bath and stored at -70°C.

The resultant preparations were about >80% pure by SDS-PAGE. These preparations had specific activities of about 10 to 20 µmol cAMP hydrolyzed per minute per milligram protein.

G. Preparation of PDE4D from *S. cerevisiae*

Yeast cells (100 g of yeast strain YI29 harboring HDUN4.11) were thawed by mixing with 150 mL glass beads (0.5 mM, acid washed) and 150 mL Lysis Buffer (50 mM MOPS pH 7.2, 10 µM $ZnSO_4$, 2 mM $MgCl_2$, 14.2 mM β-mercaptoethanol, 2 mM benzamidine HCl, 5 µg/mL each of pepstatin, leupeptin, aprotinin, calpain inhibitors I and II) at room temperature. The mixture was cooled to 4°C, transferred to a Bead-Beater®, and the cells lysed by rapid mixing for 6 cycles of

30 seconds each. The homogenate was centrifuged for 22 minutes in a Beckman J2-21M centrifuge using a JA-10 rotor at 9,000 rpm and 4°C. The supernatant was recovered and centrifuged in a Beckman XL-80 ultracentrifuge using a TI45 rotor at 36,000 rpm for 45 minutes at 4°C. The supernatant was recovered and PDE4D was precipitated by the addition of solid ammonium sulfate (0.33 g/mL supernatant) while stirring in an ice bath and maintaining the pH between 7.0 and 7.5. Thirty minutes later, this mixture was centrifuged for 22 minutes in a Beckman J2 centrifuge using a JA-10 rotor at 9,000 rpm (12,000 X g). The supernatant was discarded and the pellet was dissolved in 100 mL of Buffer A (50 mM MOPS pH 7.5, 10 μM $ZnSO_4$, 5 mM $MgCl_2$, 14.2 mM β-mercaptoethanol, 100 mM benzamidine HCl, and 5 μg/mL each of leupeptin, pepstatin, aprotinin, calpain inhibitor I and II). The pH and conductivity were corrected to 7.5 and 15-20 mS, respectively.

At a flow rate of 0.67 mL/min., the resuspended sample was loaded onto a 1.6 X 20 cm column (10 mL) of Sigma Cibacron Blue Agarose-type 300 equilibrated in Buffer A. The column was washed in succession with 50 to 100 mL of Buffer A, 20 to 30 mL of Buffer A containing 20 mM 5'-AMP, 50 to 100 mL of Buffer A containing 1.5 M NaCl, and then 10 to 20 mL of Buffer C (50 mM Tris HCl pH 8, 10 μM $ZnSO_4$, 14.2 mM β-mercaptoethanol, 2 mM benzamidine HCl). The enzyme was eluted with 20 to 30 mL of Buffer C containing 20 mM cAMP.

The PDE4D activity peak was pooled and precipitated with ammonium sulfate (0.4 g/mL enzyme pool) to remove excess cyclic nucleotide. The precipitated proteins were resuspended in Buffer X (25 mM MOPS pH 7.2, 5 μM $ZnSO_4$, 50 mM NaCl, 1 mM DTT, and 1 mM benzamidine HCl) and desalted via gel filtration on a Pharmacia PD-10® column per manufacturer's instructions. The enzyme pool was dialyzed overnight against Buffer X containing 50% glycerol. This enzyme preparation was quick-frozen in a dry ice/ethanol bath and stored at -70°C.

The resultant preparations were about >80% pure by SDS-PAGE. These preparations had specific activities of about 20 to 50 μmol cAMP hydrolyzed per minute per milligram protein.

Example 3

Phosphodiesterase Inhibition Assay

The following assay is useful for determining whether a particular compound is a PDE inhibitor. If an inhibitor compound is tested against a plurality of PDE's, it can be determined whether the compound is a specific inhibitor of a particular PDE or a non-specific inhibitor of serveral PDE's.

Phosphodiesterase inhibitor activity was determined as follows. PDE assays utilizing a charcoal separation technique were performed essentially as described in Loughney *et al., J. Biol. Chem., 271(2)*: 796-806 (1996). In this assay, PDE activity converts [^{32}P]cAMP or [^{32}P]cGMP to the corresponding [^{32}P]5'-AMP or [^{32}P]5'-GMP in proportion to the amount of PDE activity present. The [^{32}P]5'-AMP or [^{32}P]5'-GMP then was quantitatively converted to free [^{32}P]phosphate and unlabeled adenosine or guanosine by the action of snake venom 5'-nucleotidase. Hence, the amount of [^{32}P]phosphate liberated is proportional to enzyme activity. The assay was performed at 30°C in a 100 µL reaction mixture containing (final concentrations) 40 mM Tris HCl (pH 8.0), 1 µM $ZnSO_4$, 5 mM $MgCl_2$, and 0.1 mg/mL bovine serum albumin (BSA). PDE enzyme was present in quantities that yield <30% total hydrolysis of substrate (linear assay conditions). The assay was initiated by addition of substrate (32 nM [^{32}P]cAMP), and the mixture was incubated for 12 minutes. Seventy-five (75) µg of Crotalus atrox venom then was added, and the incubation was continued for 3 minutes (15 minutes total). The reaction was stopped by addition of 200 µL of activated charcoal (25 mg/mL suspension in 0.1 M NaH_2PO_4, pH 4). After centrifugation (750 X g for 3 minutes) to sediment the charcoal, a sample of the supernatant was taken for radioactivity determination in a scintillation counter and the PDE activity was calculated. The assay was performed in the absence and presence of inhibitor (various concentrations) and the IC_{50} value for the inhibitor was determined by fitting the data to a four parameter logistic dose response model.

It will be appreciated that this assay is exemplary only, and that other PDE inhibition assays can be performed as well.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those in the art, all of which are intended as aspects of the present invention. Accordingly, only such limitations as appear in the claims should be placed on the invention.

What is claimed is:

1. A method of inducing lipolyis in mammalian cells, comprising the steps of:

contacting mammalian cells with a first compound that inhibits phosphodiesterase 3 (PDE3) enzyme, and

contacting the cells with a second compound that inhibits phosphodiesterase 4 (PDE4) enzyme;

wherein the first and second compounds are administered at a concentration effective to stimulate lipolysis in the cells.

2. A method according to claim 1 wherein the cells are contacted with synergistically effective concentrations of the first and second compounds.

3. A method according to claim 1 wherein the cells are contacted with the first and second compounds simultaneously.

4. A method according to claim 1 wherein the first compound is selected from the group consisting of: amrinone, milrinone, cilostamide, anegralide, cilostazol, enoximone, piroximone, trequensin.

5. A method according to claim 1 wherein the second compound is selected from the group consisting of: rolipram, CDP840, RP-73401 D-22888 CP-80,633, SB-207499, AWD-12-281, and D-4418.

6. A method according to claim 1 wherein the first compound selectively inhibits PDE3B.

7. A composition comprising a first compound that inhibits phosodiesterase 3 (PDE3) enzyme, in admixture with a second compound that inhibits phosphodiesterase 4 (PDE4) enzyme.

8. A kit for treatment of obesity comprising a first compound that inhibits phosphodiesterase 3 (PDE3) enzyme, and a second compound that inhibits phosphodiesterase 4 (PDE4) enzyme; wherein the compounds are packaged together with instructions for administration of the compounds to a human subject to promote weight loss.

9. A kit according to claim 8 wherein the first and second compounds are in admixture in a container, and wherein the instructions are provided on a label attached to the container.

10. A method for inducing weight loss in a mammalian subject, comprising the steps of administering to the mammalian subject first and second compounds, wherein the first compound inhibits phosphodiesterase 3 (PDE3) enzyme and the second compound inhibits phosphodiesterase 4 (PDE4) enzyme, and wherein the compounds are administered at concentrations effective to promote weight loss in the mammalian subject.

11. A method according to claim 10 wherein the mammalian subject is a human subject.

12. A method according to claim 11 wherein the subject is obese.

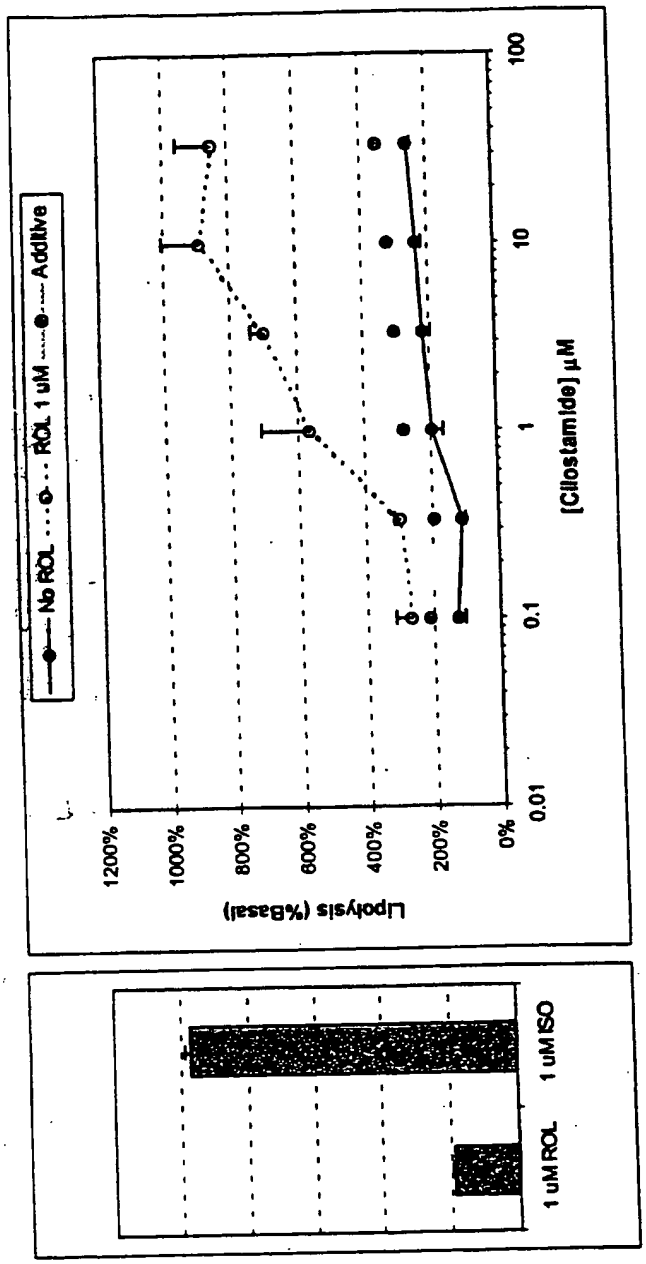
13. A method according to claim 11 wherein the first and second compounds are administered at synergistically effective concentrations.

14. A method for inducing weight loss in a mammalian subject, comprising the step of administering to the mammalian subject a composition comprising a compound that is a selective inhibitor of both a phosphodiesterase 3 (PDE3) enzyme and a phosphodiesterase 4 (PDE4) enzyme, and wherein the composition is administered at a concentration effective to promote weight loss in the mammalian subject.

15. The use of a composition according to claim 7 for the manufacture of a medicament for inducing lipolyis in cells.

16. The use of a composition according to claim 7 for the manufacture of a medicament for inducing weight loss.

Figure 1


No ROL
ROL 1 uM
Additive
Lipolysis (%Basal)
1200%
1000%
800%
600%
400%
200%
0%
0.01
0.1
1
10
100
[Cilostamide] µM
1 uM ROL
1 uM ISO

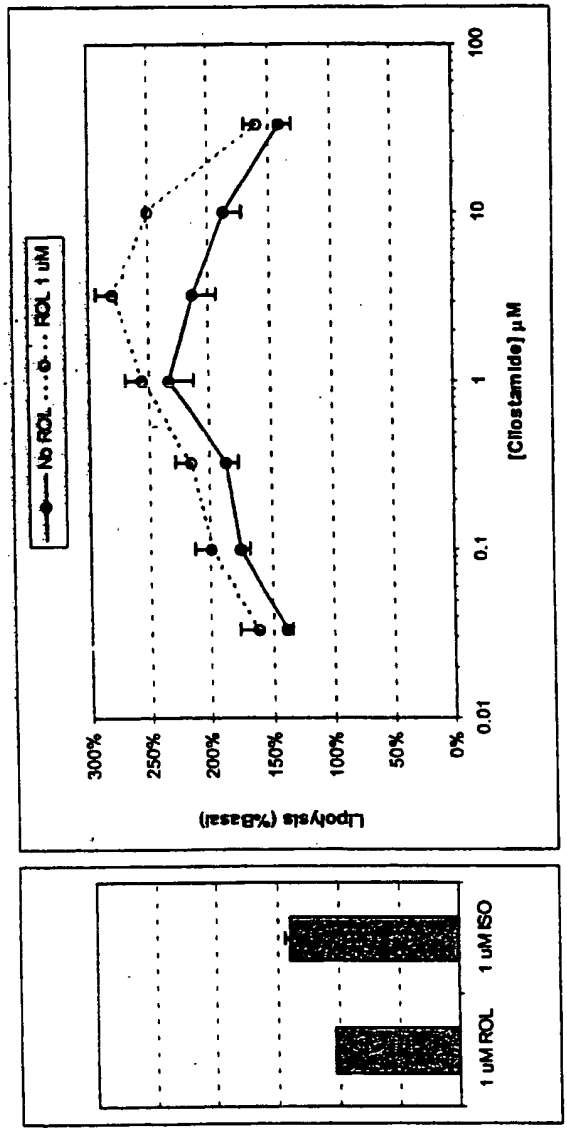

No ROL
ROL 1 uM
Lipolysis (%Basal)
300%
250%
200%
150%
100%
50%
0%
0.01
0.1
1
10
100
[Cilostamide] µM
1 uM ROL
1 uM ISO

Figure 2

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 May 2001 (25.05.2001)

PCT

(10) International Publication Number
WO 01/35979 A3

(51) International Patent Classification[7]: A61K 45/06, A61P 3/04

(21) International Application Number: PCT/US00/42137

(22) International Filing Date:
13 November 2000 (13.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/165,418 13 November 1999 (13.11.1999) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
3 January 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/35979 A3

(54) Title: COMBINED PDE3 AND PDE4 INHIBITOR THERAPY FOR THE TREATMENT OF OBESITY

(57) Abstract: The present invention provides materials and methods for the treatment of obesity that involve a combination of a PDE3 and PDE4 inhibitor in synergistically effective amounts.

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 00/42137

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K45/06 A61P3/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data, PASCAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAN X ET AL: "Synergistic interactions between selective pharmacological inhibitors of phosphodiesterase isozyme families PDE III and PDE IV to attenuate proliferation of rat vascular smooth muscle cells." BIOCHEMICAL PHARMACOLOGY, (1994 AUG 17) 48 (4) 827-35. , XP002168561	7-9
Y	* see in particular Abstract and Figure 7 *	1-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2001	05/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Isert, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 00/42137

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DOUSA M K ET AL: "Antagonists of cyclic nucleotide phosphodiesterase (PDE) isozymes PDE 3 and PDE 4 suppress lymphoblastic response to HLA class II alloantigens: a potential novel approach to preventing allograft rejection?." CLINICAL NEPHROLOGY, (1997 MAR) 47 (3) 187-9. , XP002168562 * see in particular Summary and Table 1 *	7-9
Y	SCHMITZ-PEIFFER C ET AL: "Characterization of the cyclic nucleotide phosphodiesterase isoenzymes present in rat epididymal fat cells." CELLULAR SIGNALLING, (1992 JAN) 4 (1) 37-49. , XP002168563 *see in particular Abstract; page 46; paragraph bridging pages 47,48 *	1-16
X	WO 99 14239 A (ADERMANN KNUT ;MEYER MARKUS (DE); RICHTER RUDOLF (DE); FORSSMANN W) 25 March 1999 (1999-03-25)	7-9,14
Y	*see in particular claims 1,3,24,28,33; page 11, lines 8 + *	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna al Application No
PCT/US 00/42137

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9914239 A	25-03-1999	DE 19810515 A	07-10-1999
		EP 1012188 A	28-06-2000